Atty. Dkt. No. 041673-2092

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Prussak, et al.

Title: NOVEL CHIMERIC TNF LIGANDS

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Examiner: Philip Gambel

Art Unit: 1644

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. CHARLES PRUSSAK

IN SUPPORT OF RESPONSE TO OFFICE ACTION

I, Dr. Charles Prussak, declare as follows:

1. I am a named inventor in the above-referenced patent application. I have reviewed the Office Action dated August 1, 2006 in this matter, as well as the references cited therein (PCT Application WO 98/26061, of which I am a named inventor, corresponding to U.S. Patent No. 7,070,771 (the "Kipps Application"); and, Mueller, *et al.*, *J.Biol.Chem.*, 274:38112 (Mueller, *et al.*)). My comments concerning the similarities and difference between the technology disclosed in the cited references and the present invention follow.

2. The invention relates to chimeric CD154-TNF molecules from which release of soluble TNF from cell surfaces is substantially eliminated. Use of such molecules allows one to exploit the clinically beneficial properties of TNF-α (e.g., as an anti-tumor agent) without the significant toxicities associated with soluble TNF. By way of background, tumor necrosis factor-α (TNF) is

a type-II-transmembrane protein that is cleaved by TNF- α converting metalloproteinase enzyme (TACE/ADAM-17) to release soluble TNF, a cytokine that can cause serious systemic toxicity. Human cells transfected with vectors encoding TNF without the TACE site (ΔTACE-TNF) still release substantial amounts of functional TNF at levels that vary by cell type. In contrast, chimeric CD154- TNF molecules of the invention direct high-level surface expression of a functional TNF that is resistant to cleavage in all cell-types. The chimera are also more effective than TNF in inducing regression of established, syngeneic tumors in mice, without the toxicity associated with soluble TNF.

3. The Kipps Application describes general characteristics of TNF family ligand chimera, from which a metalloproteinase cleavage site may be removed. However, Mueller, *et al.* confirms that deletion of the cleavage site alone is not sufficient to substantially eliminate soluble TNF release; i.e.; from cells expressing non-chimeric TNF lacking the TACE-cleavage-site (Mueller, *et al.*, at 381777, first paragraph). Therefore, it is my opinion that, in combination, the references suggest that removal of a cleavage site from a TNF family ligand chimera would not be sufficient to stabilize the molecule when expressed on a cell membrane or prevent release of soluble TNF.

4. , Although removal of the TACE-cleavage site in the TNF molecule can reduce cleavage, our data indicate that release of soluble TNF from such molecules likely occurs because the molecule is apparently cleaved at other extracellular sites as well. In contrast, chimeric TNF and ligand polypeptides of the invention have the proximal extracellular domain (III) from CD154 domain III joined directly or indirectly to the distal extracellular domain (IV) of TNF, thereby removing the TACE-cleavage site from these domains which substantially eliminates release of soluble TNF, notwithstanding the putative presence of other cleavage sites. Not only was this a surprisingly discovery, we have also learned that the virtual elimination of soluble TNF release from the chimeric molecules is achievable in a variety of cell types. In contrast, reduction in soluble TNF release from non-chimeric TNF molecules lacking the cleavage site (per Mueller, *et al.*) does not substantially reduce soluble TNF release in many cell types (see, e.g., Table I below—reduction of soluble TNF release achieved to a substantial extent only in a HT1376 bladder carcinoma cell line).

5. The data discussed herein were generated by myself and colleagues using chimeric forms of TNF that juxtaposed the active ligand-binding portion of the TNF molecule with the transmembrane and proximal extracellular domains of TNF family protein CD154. In the chimeric molecules, the TACE-cleavage site was removed from the proximal extracellular domain of CD154 (Domain III) and from the distal extracellular domain of TNF (Domain IV). More particularly, the human CD154-TNF chimeric constructs were generated as follows: DNA encoding a fragment of human CD154 spanning the intracellular, transmembrane, and partial extracellular region adjacent to the transmembrane region of CD154 was amplified from the full length CD154 cDNA by PCR using the following primers (5' GAC AAG CTT ATG ATC GAA ACA TAC AAC C and 5' TCA GGA TCC TCA TCT TTC TTC G). EcoRI and BamHI restriction enzyme sites were added at the 5' and 3' ends of this fragment. DNA encoding the extracellular region of human TNF distal to the TACE cleavage site was also PCR amplified from the full length TNF cDNA.

6. We examined cells infected with the chimeric constructs [wild-type TNF (Ad-wtTNF), or TNF lacking the defined TACE site (Ad-ΔTACE-TNF)] for expression of functional cell-surface TNF relative to that of the soluble cytokine. The cells utilized were: HeLa (human cervical carcinoma), HT1080 and A549 (human lung), HCT-15 and COLO-205 (human colon), DU-145 (human prostate), RPMI-8226 (human myeloma), HT1376 (human bladder), L929 (mouse fibroblast), WEHI-164 (mouse fibrosarcoma) and A20 (mouse B cell lymphoma), each obtained from ATCC. 293AC2, a subclone of 293, was obtained from Molecular Medicine Inc. (San Diego, CA). All cell lines except for 293AC2 were cultured in RPMI-1640 containing 10% fetal calf serum and 2mM l-glutamine and maintained in a 5% CO_2 incubator. 293AC2 cells were cultured in DMEM containing 10% fetal calf serum and 2mM l-glutamine and maintained in a 10% CO_2 incubator.

8. Our results showed that cells transfected with Ad-ΔTACE-TNF unexpectedly released substantial amounts of functional TNF, albeit at levels that were significantly less than that of cells transfected with pwtTNF. The culture supernatants of HT1080 cells transfected with either

Ad-wtTNF or Ad-ΔTACE-TNF (but not control pcDNA3) induced apoptosis of L929 cells, a TNF-sensitive mouse fibroblast cell line (Fig. 1a [all figures referenced are appended]).

9. Upon further investigation into the expression of ΔTACE-TNF, we found soluble TNF was released from various human cell lines infected with adenovirus encoding ΔTACE-TNF (Ad-ΔTACE-TNF) (Table 1, below). Regardless of the cell line used, we found that soluble TNF released by Ad-ΔTACE-TNF infected cells, albeit at significantly lower levels ($P < 0.05$, Student's t test) than that released from Ad-wtTNF infected cells (Table 1). No detectable TNF was produced from any of the cell lines prior to transduction or after transduction with a control adenovirus vector.

Table 1. Soluble TNF Generation Following Ad-TNF Infection

Name	Type	Soluble TNF*		
		wtTNF	□TACE-TNF	CD154-TNF
HT1080	Lung	43,100 ± 3,000	4,000 ± 100	< 40
A549	Lung	48,200 ± 2,500	14,200 ± 900	N.D.
HeLa	Cervical	640,000 ± 600	52,000 ± 500	< 40
RPMI-8226	Myeloma	21,400 ± 1,600	4,500 ± 200	< 40
HT1376	Bladder	60,200 ± 300	300 ± 10	< 40

* (pg/ml ± s.d.) N.D. = not determined. Cell lines were infected at an MOI of 10 for two days followed by analysis of soluble TNF by ELISA.

10. Interestingly, the difference in the amounts of soluble TNF released by cells expressing comparable levels of wtTNF versus ΔTACE-TNF varied for the different cell lines tested, from an approximate three-fold difference for the lung cell line A549 to greater than a 200-fold difference for the bladder cell line HT1376. Furthermore, addition of the broadly active MMP inhibitor GM6001 to HeLa cells transfected with Ad-ΔTACE-TNF did not prevent these cells from releasing soluble TNF (11,000 pg/ml ± 100 with inhibitor versus 52,000 pg/ml ± 500 without, mean ± S.E., n = 3). Collectively, these studies indicate that ΔTACE-TNF still has a cleavage site(s) that allows for release of soluble TNF from cells transduced with this truncated

form of TNF (in contrast to chimeric molecules of the invention that lack the TACE-cleavage site from the proximal extracellular domain [CD154] and distal extracellular domain [TNFα]).

11. We also compared the generation of soluble TNF by cells infected with Ad-CD154-TNF to cells infected with either Ad-wtTNF or Ad-ΔTACE-TNF (Fig. 1b). To control for nonspecific TNF secretion by cells, we also examined cells that either were infected with control adenovirus encoding the irrelevant transgene ß-galactosidase (Ad-LacZ) or that were not infected (noninfected). We found that Ad-CD154-TNF infected HT1080 cells produced significantly less soluble TNF (< 40 pg/ml, below detection limit of ELISA assay, $P < 0.05$, Student's t test) than cells infected with adenovirus encoding either wtTNF (43,100 pg/ml ± 3,000 pg/ml) or ΔTACE-TNF (4,000 pg/ml ± 100 pg/ml) (Table 1). Infection of other cell lines with Ad-CD154-TNF, including HCT-15 and COLO-205 (human colon), DU-145 (human prostate), and 293AC2 (human kidney) generated cells that had high-level cell-surface expression with negligible release of soluble TNF.

12. We also examined for the cell-surface expression of TNF on cells transduced with Ad-wtTNF, Ad-ΔTACE-TNF, or Ad-CD154-TNF (Fig. 1c). In addition, we analyzed cells infected with adenovirus encoding a different chimeric molecule composed of the TNF extracellular domain fused to the amino-terminal portion of another TNF family ligand molecule, CD70 (Ad-CD70-TNF. The CD70-TNF chimera was comprised of a shorter extracellular stalk region (5 amino acids) than CD154-TNF (65 amino acids), to determine if expression results were specific for CD154-TNF. At MOI ratios of 1 to 10, Ad-CD154-TNF-infected cells expressed higher cell-surface levels of TNF than did cells infected with adenovirus encoding any one of the other Ad-TNF constructs (Fig. 1c, and data not shown). Indeed, although the short stalk domain of CD70-TNF, a construct that we hypothesized should also anchor TNF to the plasma membrane, apparently resisted proteolytic cleavage, we could not effect high-level surface expression of CD70-TNF on cells infected with Ad-CD70-TNF, even at high MOI. This indicates that the composition (e.g., CD154 versus CD70) rather than the length of the stalk region may be important in governing the relative level of cell-surface expression produced by TNF ligand chimera of the invention, a concept not suggested in the cited references.

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13. In addition, we performed linear regression analyses on the relationship between released TNF and cell-surface TNF observed for cells infected at various MOI with each of the Ad-TNF constructs. In each case, we could define a linear relationship between the relative expression levels of soluble TNF versus cell-surface TNF for each cell type following infection at various MOI. We compared the different Ad-TNF constructs (including a CD70-TNF chimera) by calculating the slope of the line that defined each relationship, with steeper slopes indicating production of greater amounts of soluble cytokine relative to that of cell-surface TNF. We found that the relative steepness of slopes for the cells infected with each of the Ad-TNF constructs had the following relationship: Ad-wtTNF > Ad-ΔTACE-TNF > Ad-CD70-TNF > Ad-CD154-TNF. For example, the slopes for HT1080 cells infected with the Ad-TNF constructs were as follows: Ad-wtTNF (slope = 1,300; $R^2 = 0.8$) > Ad-ΔTACE-TNF (slope = 290; $R^2 = 0.8$) > Ad-CD70-TNF (slope > 15; $R^2 = 0.9$) > Ad-CD154-TNF (slope = 0; $R^2 > 0.9$) (Fig. 1d). Likewise, the slopes for infected HeLa cells were as follows: Ad-wtTNF (slope = 4,600; $R^2 > 0.9$) > Ad-ΔTACE-TNF (slope = 46; $R^2 > 0.9$) > Ad-CD70-TNF (slope > 10; $R^2 > 0.9$) > Ad-CD154-TNF (slope = 0; $R^2 > 0.9$) (Fig. 1d).

14. The linear relationships described in the preceding paragraph are consistent with the values reported for the fold difference in amount of soluble TNF released from cell lines infected with Ad-wtTNF compared to cells infected with either Ad-ΔTACE-TNF or Ad-CD154-TNF (Table 1). Infection with Ad-CD154-TNF resulted in high-level surface expression of TNF with negligible release of soluble TNF, resulting in a slope of zero (Fig. 1d). As such, CD154-TNF represents a novel type of TNF that, in contrast to ΔTACE-TNF, can be expressed on the cell-surface of a variety of different cell types without detectable release of TNF.

15. To examine whether the chimeric CD154-TNF construct was functional we co-cultured TNF-sensitive L929 cells with HeLa cells that were infected with adenovirus encoding either wtTNF or CD154-TNF (Fig. 2). We separated the L929 and HeLa cells from direct cell-cell contact using a 0.4 μ transwell insert. As such, apoptosis of L929 should only result from diffusion of soluble TNF from the HeLa cells across the membrane to the L929 cells. As

expected, Ad-wtTNF infected HeLa induced apoptosis of L929 even when separated by a transwell membrane. In contrast, Ad-CD154-TNF infected HeLa cells did not induce apoptosis of L929 cells more than did control-infected HeLa cells when separated from the L929 cells across the transwell membrane (Fig. 2). The observed activity was due to TNF signaling because the killing of L929 cells could be blocked specifically by adding a neutralizing mouse anti-human TNF mAb to the culture medium prior to the assay.

16. In summary, although cells transduced with Ad-ΔTACE-TNF (i.e., lacking a metalloproteinase cleavage site per Mueller, *et al.*) released less soluble TNF than did cells transduced with Ad-wtTNF, some transduced cells still released significant amounts of soluble cytokine (e.g. A549, Table 1). Because the TNF released by Ad-ΔTACE-TNF-transduced cells could induce apoptosis of cells sensitive to TNF-mediated apoptosis (Fig. 1a), we conclude that TNF does have sites other than the TACE cleavage site that can be cleaved to release a functional soluble cytokine. Removal of the cleavage site alone, per Mueller, *et al.* is clearly insufficient to substantially eliminate release of soluble TNF in any one cell type, much less all cell types. Nor would elimination of the cleavage site from the extracellular proximal *or* distal domains of a chimera be sufficient. The site must be eliminated from both domains, and the domains drawn from CD154 (rather than other TNF family ligands, such as CD70) and TNF, to achieve the level of soluble TNF elimination in all cell types tested, as demonstrated in the results discussed above.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: Dr. Charles Prussak

Oct 7th, 2006

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Figure 1

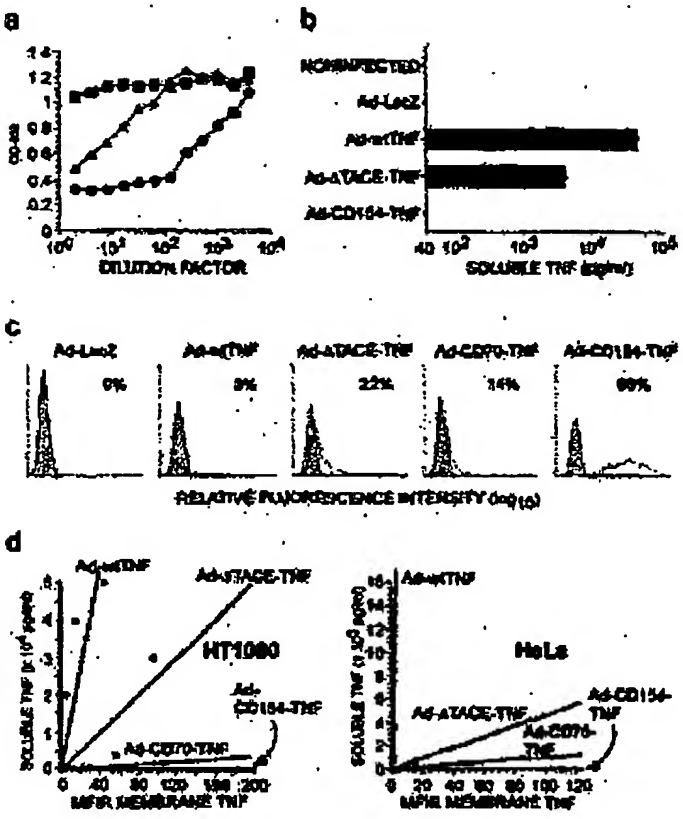

Figure 1. Expression of TNF by transfected cells. (A) Secretion of soluble TNF from ΔTACE-TNF transfected cells. HT1080 cells were transfected with pcDNA3 plasmids encoding vector alone (square), wtTNF (circle), or ΔTACE-TNF (triangle) and incubated for two days. Serial dilutions of cell supernatants were then incubated with L929 cells and soluble TNF bioactivity determined by the measurement of L929 apoptosis using the XTT colorimetric assay. (B) HT1080 cells were infected with adenovirus as indicated for two days. The cell supernatant was then analyzed for soluble TNF by ELISA. (C) Cell-surface expression of TNF by Ad-infected HeLa cells. HeLa cells were infected with the indicated adenovirus and then analyzed for TNF surface expression by flow cytometry (open histograms). Shaded histograms represent cells stained with isotype control antibody. The percentage of surface-TNF-positive cells is indicated for each histogram. (D) The relationship of soluble TNF versus the mean fluorescence intensity ratio (MFIR) of TNF surface expression was plotted for HT1080 cells (left graph) and HeLa cells (right graph) infected at increasing MOI with adenovirus encoding wtTNF (closed squares), ΔTACE-TNF (open squares), CD70-TNF (open triangles), and CD154-TNF (closed circles). Data points from four different MOI for each TNF construct were used to determine the linear functions plotted on the graph.

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Figure 2

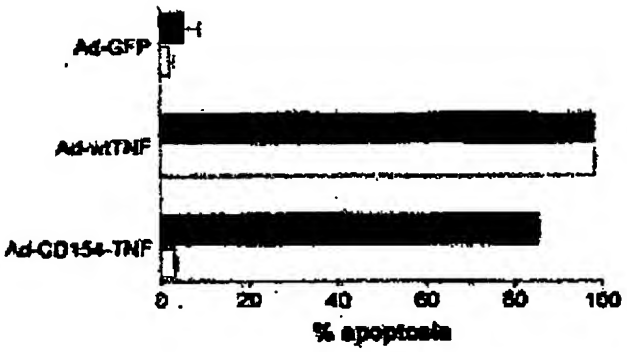

Figure 2. In vitro and in vivo toxicity of soluble and cell-surface TNF. Contact-dependent killing of L929 cells by Ad-TNF infected HeLa cells. HeLa cells were infected with adenovirus encoding the indicated transgene (GFP, wtTNF, or CD154-TNF) and then stained with CFSE and plated out with an equal number of L929 cells, either to allow cell-cell contact (co-culture, filled bars) or HeLa cells were placed in the upper well of a transwell filter insert (trans-well, empty bars) to prevent direct cell-cell contact with L929 cells. Following 18 hr co-culture, L929 cells were analyzed for viability by flow cytometry.